Molecular Mechanism of Quinclorac Resistance in Multiple-Herbicide Resistant Echinochloa phyllopogon

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INTRODUCTION

Weeds have been controlled by using synthetic chemicals since 1945. The first commercially available herbicide was 2,4-dichlorophenoxyacetic acid (2,4-D) which is a synthetic auxin (Peterson *et al.*, 2016). Since then, numerous compounds with various modes of action have been developed for crop protection and applied worldwide. The long history of herbicide use has selected for weed populations with multiple herbicide resistance (Heap, 2019). Mechanisms include target-site resistance (TSR) and non-target-site resistance (NTSR) (Powles & Yu, 2010). TSR is characterized by alterations in the gene(s) encoding the herbicide target protein. These include overproduction of target site proteins or substitution of amino acids which change the herbicide binding site structure. NTSR is characterized by other resistance mechanisms such as reduced herbicide penetration and translocation, enhanced herbicide degradation, and protection against herbicide collateral damage (Délye, 2013). Multiple herbicide resistance may result from the accumulation of numerous mechanisms after sequential herbicide selection or resistance alleles by gene flow, especially in outcrossing species (Beckie & Tardif, 2012). Multiple herbicide resistance may also occur by a single NTSR mechanism such as enhanced herbicide stress tolerance or metabolism (Yu & Powles, 2014). The latter mechanisms confer herbicide resistance to populations heretofore unexposed to the agent. Although its threat to agriculture is substantial, our current knowledge of NTSR-mediated multiple herbicide resistance is limited.

Echinochloa phyllopogon (Stapf) Koss. (late watergrass) is a self-pollinating allotetraploid. It is a major weed in rice fields and may cause serious crop yield loss if left unchecked (Yamasue, 2001). In 1997, the first case of herbicide resistance in *E. phyllopogon* was discovered in Sacramento Valley, California, U.S.A.(Fischer *et al.*, 2000a). This population exhibited resistance to the herbicides used for selection (thiocarbamates and fenoxaprop-ethyl) and others such as acetolactate synthase (ALS) inhibitors, acetyl-CoA carboxylase (ACCase) inhibitors, clomazone, and quinclorac (Fischer *et al.*, 2000b; Osuna *et al.*, 2002; Ruiz-Santaella *et al.*, 2006; Bakkali *et al.*, 2007; Yasuor *et al.*, 2009, 2012), most of which were uncommercialized at the time. Attempts to elucidate the mechanisms of multiple herbicide resistance led to the identification of overexpressing herbicide-metabolizing cytochrome P450 (P450) genes in resistant plants (Iwakami *et al.*, 2014a). The P450s CYP81A12 and CYP81A21 metabolize ALS inhibitors,

ACCase inhibitors, and clomazone, therefore confer multiple herbicide resistance (Guo *et al.*, 2019; Iwakami *et al.*, 2019)

Quinclorac is a synthetic auxin herbicide with a quinolinecarboxylic acid backbone known to effectively control Poaceae weeds such as *Echinochloa* spp. As with other synthetic auxins, quinclorac is recognized by the F-box protein of the TIR1/AFB family of auxin receptors and a member of the Aux/IAA family of co-repressor proteins (Villalobos *et al.*, 2012; LeClere *et al.*, 2018). Consequently, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and other auxin-responsive genes are induced. In turn, ACC is biosynthesized then oxidized by ACC oxidase to ethylene and hydrogen cyanide (HCN). High HCN levels inhibit several vital plant metabolism enzymes and may result in death in quinclorac-sensitive plant species (Fig. 1) (Grossmann, 2010).

Multiple herbicide resistant *E. phyllopogon* exhibits resistance to quinclorac even though the resistance was never selected for by quinclorac in the fields. Previously, quinclorac resistance was characterized in multiple herbicide resistant *E. phyllopogon* (Yasuor *et al.*, 2012). The authors reported diminished ethylene production and enhanced activity of the cyanide detoxifying enzyme β-cyanoalanine synthase (β-CAS). They concluded that these two mechanisms, which are independent of P450 overexpression, participate in quinclorac resistance. In the present study, the role of β-CAS in quinclorac resistance was investigated using the progeny of crossed resistant (R) and susceptible (S) *E. phyllopogon* lines. Our results did not support the longstanding hypothesis that β-CAS activity accounts for decreased quinclorac sensitivity in plants. In contrast, it was revealed that quinclorac resistance is associated with reduced ethylene production and enhanced herbicide metabolism in the progeny. The data presented herein will be the basis for elucidating the molecular mechanisms underlying quinclorac resistance in *E. phyllopogon*, and also clarifying heretofore poorly understood plant auxin responses.

SUMMARY

Herbicide is one of the most important tools for crop protection in modern agricultural system. Under stress from recurrent used of herbicide, weed resistant to herbicide has been increasing worldwide due to evolutionary adaptation. The failure to control weeds owing to herbicide resistance has become problematic issue and resistance mechanisms study is urgently required. *Echinochloa phyllopogon* is a noxious weed in rice cultivation causing a large decreased of rice yield if uncontrolled. *E. phyllopogon* populations in California have evolved resistance to multiple herbicides with different modes of action including quinclorac. Quinclorac is an auxin herbicide which induces production of the senescence hormone ethylene, resulting in the accumulation of toxic HCN. Yasuor *et al.* (2012) revealed enhanced activity of the HCN-detoxifying enzyme β-cyanoalanine synthase (β-CAS) in resistant *E. phyllopogon*. Therefore, enhanced activity of the enzyme has been considered as one of the mechanisms of quinclorac resistance in this biotype.

In this study, the involvement of β-CAS in quinclorac resistance was examined at the molecular level. *β-CAS* genes were isolated from resistant (R) and susceptible (S) plants. Two copies of the gene were found: *β-CAS1* and *β-CAS2*. Accumulation of *β-CAS1* mRNA in R plants was five-fold higher than in S plants, while no difference was observed in that of *β-CAS2*. Alternative splicing leading to aberrant transcripts was observed in *β-CAS1* of the S line whereas only normal transcripts of *β-CAS1* existed in the R line. It was inferred that a single nucleotide polymorphism (SNP) on the border of an exon-intron junction explains the loss of alternative splicing in the R line. The association of the SNP, existence of aberrant transcripts, transcript level, and enzyme activity of β-CAS1 were investigated in 32 lines of F6 crossed progeny of S and R plants. Complete correlation among four characteristics was observed. The results suggest that enhanced β-CAS activity in the R line is caused by the loss of aberrant alternative splicing. However, no correlation was observed between the enhanced β-CAS activity and quinclorac resistance in F6 lines, indicating the enhanced β-CAS activity is not involved in quinclorac resistance in this biotype. Moreover, the transgenic Arabidopsis overexpressing *β-CAS1* from *E. phyllopogon* barely showed resistance to quinclorac. The *β-CAS* knockout rice which lost β-CAS activity responded to quinclorac in the similar way as wild-type rice, clearly negated the involvement of β-CAS in plant quinclorac sensitivity.

Reduction of ethylene production was observed in R line after quinclorac application. The reduced ethylene was perfectly co-segregated with quinclorac resistance in F6 lines, suggesting that the mechanism that mitigates ethylene production plays a major role in quinclorac resistance in *E. phyllopogon*. Previous study revealed that two cytochrome P450s, CYP81A12 and CYP81A21, conferred resistance to *acetolactate synthase (*ALS)-inhibitors in *E. phyllopogon* which exhibits resistance to multiple herbicides including quinclorac (Iwakami *et al.*, 2014a). Therefore, further analyses were conducted to examine whether quinclorac resistance associated with enhanced herbicide metabolism by the P450s. Interestingly, complete correlation between quinclorac resistance and up-regulation of *CYP81A12* and *CYP81A21* genes in F6 lines were found. Arabidopsis carrying either of the P450s grew better than wild type in MS medium containing quinclorac. Moreover, reduced ethylene production was observed in the transgenic Arabidopsis, indicating that overexpression of *CYP81A12* and *CYP81A21* conferred quinclorac resistance through the P450-mediated metabolism leading to reduced stimulation of the ethylene production pathway. The resistant mechanism affects cross-resistance to other auxin herbicides in three different chemical classes: 2,4-D, NAA, and picloram. Metabolism study using heterologous expression in *E. coli* revealed that not only CYP81A12 and CYP81A21 but also CYP81A24 were able to metabolize quinclorac. Arabidopsis overexpressing either of the P450s exhibited tolerance to 2,4-D and NAA as well. However, picloram could not be metabolized by any CYP81As.

Overall, this research revealed the 20 year-long mystery of herbicide resistance in the noxious weed. The research provides more understanding about resistance evolution and will be useful for weeds management in the future. Further research is required to show the role of P450s in quinclorac metabolism in detail. Since P450s have potential to metabolize many herbicides, more understanding in metabolizing mechanism benefits the further utilization of P450s.

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